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Quantification of SMN protein in leucocytes from spinal muscular atrophy patients: effects of treatment with valproic acid

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ABSTRACT

Background Spinal muscular atrophy (SMA) is caused by the homozygous deletion of the survival motor neuron (*SMN1*) gene. The nearly identical *SMN2* gene produces small amounts of full-length mRNA and functional SMN protein, due to a point mutation in a critical splicing site. Increasing SMN protein production by histone deacetylase inhibiting drugs such as valproic acid (VPA) is an experimental treatment strategy for SMA.

Objective To investigate whether an SMN-specific ELISA could detect changes in SMN protein expression in peripheral blood mononuclear cells (PBMCs) after treatment with VPA.

Methods The authors developed a sensitive SMN-specific ELISA. Six patients with SMA types 2 and 3 participated in the study. Recombinant SMN calibration curves were used to calculate SMN protein levels in PBMCs before and after 4 months of VPA treatment.

Results The SMN ELISA was able to detect small differences in SMN protein concentrations, and differences in SMN protein levels in Epstein–Barr virus immortalised lymphocyte cell lines from SMA type 1 and 2 patients, carriers and healthy individuals ($p < 0.05$). The mean SMN protein level in PBMCs from SMA patients was 22% (SD 15%) of the value in a healthy control. VPA treatment resulted in significantly increased SMN protein levels in five out of six SMA patients compared with baseline values ($p < 0.05$), but did not restore SMN levels to normal values.

Conclusions SMN protein quantification by this SMN ELISA is a useful additional tool for evaluating the effects of experimental treatment in SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disease caused by a homozygous deletion of the survival motor neuron (*SMN1*) gene.¹ The nearly identical *SMN2* gene produces only low levels of full-length SMN mRNA due to a point mutation in a critical splicing site in exon 7. SMA severity inversely correlates with *SMN2* copy number, and *SMN2* copy number correlates with the amounts of full-length SMN mRNA and protein.^{2–3} Histone deacetylase (HDAC) inhibitors, such as valproic acid (VPA), increase full-length SMN mRNA and SMN protein in cell lines.^{4,5} VPA treatment also increases SMN mRNA levels in peripheral blood mononuclear cells (PBMC) of SMA patients and carriers.⁶ Because SMN protein level quantification techniques may be useful tools for

clinical trials, we developed an SMN-specific ELISA to measure SMN protein levels in small volumes of blood.⁷ The ELISA can detect small changes in SMN concentrations, and can be used to monitor effects of experimental treatment.

MATERIALS AND METHODS

Patients

Patient characteristics are summarised in the table 1. *SMN2* copy numbers were determined as described elsewhere.⁸ Parents of six SMA patients (no 1–6) requested their treating physicians to prescribe VPA while awaiting results from an ongoing clinical trial.⁹ The medical ethics committee of the Academic Medical Centre (Amsterdam, The Netherlands) approved of compassionate use of VPA. All parents were informed of possible side effects and hazards of treatment with VPA, and all parents gave written informed consent prior to treatment. Only patients with normal aminotransferase values were included. One patient (no 7) was not treated with VPA.

VPA (Depakine syrup 60 mg/ml) was initiated at a dose of 20 mg/kg and administered twice daily. This dose was gradually increased until a plasma level within the therapeutic range was reached (70–100 mg/ml). At morning control visits (baseline and 13 weeks after start of treatment), body weight, aminotransferase and serum creatinine levels, VPA plasma levels, and SMN protein concentrations in lymphocytes were determined. SMN protein levels in PBMCs from patient 2 were determined at baseline and each month until 3 months after start of treatment.

PBMCs from four healthy blood donors (HBD) were obtained and isolated at 10:00 with an interval of 4 weeks and on 1 day at 10:00 and 16:00 to determine the variation in SMN expression. All HBD had two *SMN1* copies and one to three *SMN2* copies. Protein extracts from one of these healthy donors was used as reference in experiments that were performed to determine the effect of VPA treatment in SMA patients.

Cell isolation and protein extraction

PBMCs were isolated from 5 ml EDTA-blood samples using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and lysed with RIPA buffer (150 mM NaCl, 1% triton, 20 mM Tris–HCl, pH 7.5–7.8, 0.5% deoxycholate, 0.1% SDS, 10 mM EDTA (pH 8)). Lysates were stored at -80°C .

Table 1 Characteristics of spinal muscular atrophy (SMA) patients

No	Sex	SMA type	Age at inclusion	SMN2 copy no
1	F	3A	11.3	3
2	F	3A	5.9	3
3	F	2	3.0	3
4	M	2	4.8	3
5	F	2	16.5	3
6	M	2	1.6	3
7	M	3A	5	3

Age at inclusion in years. All patients had a homozygous deletion of the survival motor neuron (*SMN1*) gene.
F, female, M, male.

Protein concentrations were measured using a D_C Protein Assay (Bio-Rad, Hercules, California).

Immortalised cell lines from SMA patients

PBMCs from five additional SMA patients, two heterozygous carriers and one HBD were isolated from whole blood by Ficoll density centrifugation and resuspended in 10 ml RPMI medium containing 1 ml Epstein–Barr virus (EBV), 1 µg/ml of phytohaemagglutinin, 10% fetal bovine serum and 1% penicillin/streptavidin. Cells were incubated at 37°C with 5% CO₂ and used as additional tools to validate the ELISA.

ELISA for quantification of SMN protein

Ninety-six-well plates (NUNC, Maxisorp, Roskilde, Denmark) were incubated overnight with 50 µl of 20 µl/ml monoclonal murine IgG1 antihuman SMN (BD Biosciences, Pharmingen, San Diego, California) in PBS at 4°C. After washing with PBS, plates were blocked with 200 µl PBS 10% casein (Vector, Burlingame, California) for 1 h. For calibration experiments, a range of PBMC protein and recombinant SMN (rSMN) (Abnova, Taipei, Taiwan) concentrations were added in triplicate and were incubated for 2 h at room temperature. Fifty microlitres of 150 µg/ml PBMC protein in PBS allowed quantification in the linear part of the calibration curve, and was thereafter used for all experiments. SMN concentrations in samples obtained before and after the start of VPA treatment and a healthy control as reference were measured simultaneously in one ELISA plate. rSMN calibration curves were used to calculate equivalent SMN protein concentrations in PBMCs. After washing, wells were incubated with 50 µl of PBS–2% casein containing 25 µl/ml of polyclonal rabbit IgG antihuman SMN (Santa Cruz Biotechnology, Santa Cruz, California) and incubated for 1 h at room temperature. After washing, wells were incubated with peroxidase-conjugated goat antirabbit antisera (Pierce, Rockford, Illinois) for 1 h at room temperature and developed using TMB (Tebu-Bio, Boechout, Belgium). OD values were determined at 450 nm using an ELISA reader (Bio-Rad).

Statistics

An independent t test was used to compare SMN protein values between patients, carriers and a healthy donor. A paired sample t test was used to analyse differences before and after treatment with VPA. p Values ≤0.05 were considered significant.

RESULTS

Quantification of SMN protein

The ELISA could detect SMN protein (both recombinant and from PBMCs) concentrations from 25 ng/ml. OD values linearly increased from 25 ng/ml to 300 ng/ml. The day-to-day variation

in SMN expression in PBMCs from healthy blood donors was 10% (SD 4.8%). Increases of more than 20% (mean variation ±2SD) of the SMN-specific signal in patients treated with VPA were, therefore, considered to be a treatment effect. SMN expression in immortalised lymphocytes from patients with SMA types 1 and 2, but not from an SMA type 3 patient carrying five *SMN2* copies, were lower compared with SMA carriers and HBD (p<0.05).

VPA increases SMN protein expression in SMA patients

The mean SMN protein expression in PBMCs from SMA patients at baseline was 22% (SD 15%) and at follow-up (during VPA treatment) 30% (SD 12%) of levels in a sample from a HBD. VPA treatment resulted in increased SMN protein expression (ie, >2SD) in five SMA patients (patients 1–5) (p<0.05). The increase in concentration ranged from 27 to 289% compared with baseline values (figure 1). The mean SMN protein level in PBMCs from patient 2 was 149% after 1 month, 196% after 2 months and 220% after 3 months of VPA treatment compared with baseline.

Side effects

VPA was well tolerated, and no serious adverse events were reported.

DISCUSSION

Open label studies have reported a functional improvement of some SMA patients after treatment with VPA,^{9 10} but the selection of meaningful clinical outcome measures to determine efficacy of experimental therapy in SMA is complicated by the very slow progression following onset.^{9 11 12} SMN quantification techniques may therefore be helpful additional tools to investigate experimental treatment effects. The feasibility of the ELISA was confirmed by the finding that experimental day-to-day variation in SMN expression was low, and that the technique can detect relatively small differences in SMN concentrations.

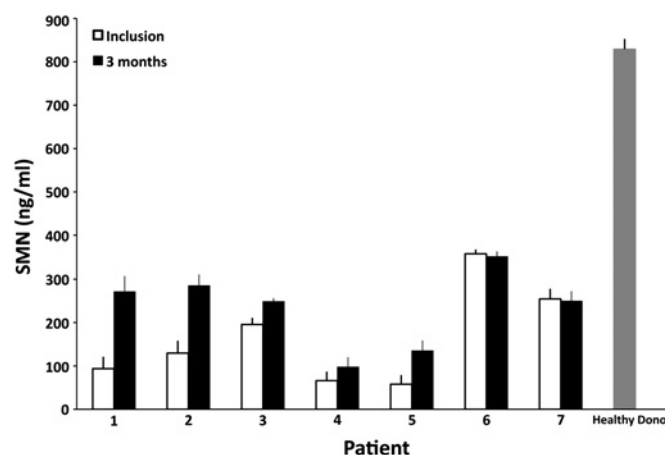


Figure 1 Survival motor neuron (SMN) protein expression at inclusion and after 3 months of valproic acid (VPA) treatment. Data are presented as mean recombinant SMN (ng/ml) concentration (+SD) from one of three representative experiments. VPA treatment resulted in increased SMN protein expression in five spinal muscular atrophy (SMA) patients (patients 1–5) (p<0.05). SMN protein expression after VPA treatment remained stable in one patient (patient 6). VPA expression did not change in an SMA patient without VPA treatment (patient 7). The grey bar reflects the SMN concentration in peripheral blood mononuclear cells from a representative healthy donor (two *SMN1*, one *SMN2* copies).

Short report

SMN protein quantification may be more relevant than mRNA levels.² PBMCs are easy to obtain, and the variation in lymphocytes and monocytes in PBMCs fractions does not influence SMN concentrations.¹³ The use of rSMN calibration curves allows results to be compared from different plates and screening of large numbers of patients. SMN-specific ELISA is easier to use than other techniques such as western blot.⁷

In contrast to previous studies which reported reduced SMN protein levels in leucocytes from SMA type 1 patients but almost normal levels in leucocytes from SMA type 2–3 patients,^{2,3} SMN concentrations were reduced in PBMCs from all patients with SMA type 2 and 3a. This may be explained by differences in methodology, or indicate that other factors than *SMN2* copy number, such as epigenetic effects, critically determine transcription of the *SMN2* gene.^{14,15} Although reduced SMN concentrations could also be detected in EBV immortalised lymphocytes from SMA patients, the difference in SMN concentrations between carriers and HBD was smaller than in PBMCs. This may be attributed to effects of cell culture,⁷ or as yet unknown mechanisms induced by immortalisation.

Previous studies showed that HDAC inhibiting drugs such as VPA increase SMN mRNA and protein concentrations in lymphoid cell lines and fibroblasts.^{4,5} VPA treatment also increases SMN mRNA levels in lymphocytes of patients and carriers, and increases levels of SMN protein in carriers.⁶ Our results suggest that VPA treatment also increases SMN protein concentrations in the majority of SMA patients. Importantly, VPA treatment did not result in restoration of normal SMN protein levels, and the effect size differed considerably between patients, despite the fact that all patients carried three *SMN2* copies. Patients responding to VPA treatment had lower baseline SMN protein concentrations compared with the non-responder. This may suggest that *SMN2* copy number does not determine effect size or responsiveness to VPA treatment.

Quantification of SMN concentrations may be a useful tool to evaluate efficacy of experimental treatment, such as more potent HDAC inhibitors. It can help to identify (non)-responders, to determine whether increasing SMN concentrations is a strategy

that leads to clinically meaningful effects and, if so, which increase in SMN expression would alter SMA disease course.²

Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was provided by the Ethics Committee of the Academic Medical Centre Amsterdam, The Netherlands.

Provenance and peer review Not commissioned; externally peer reviewed.

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